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(54) Title: SULPHUR CONTAINING DINUCLEOTIDE PHOSPHORAMIDITES (57) Abstract There is provided a process for the solid phase synthesis of phosphorothioate oligonucleotides in which a dimeric phosphoramidite synthon is used to extend the oligonucleotide chain, the synthon having an optionally protected thioester group in its internucleotide linkage. Novel dimeric phosphoramidite synthons having such a thioester group are also described. The process enables increased yield of the oligonucleotide of interest with enhanced separation from impurities. The presence of the thioester linkage stabilises the oligonucleotide end product, facilitating its use as an anti-sense oligonucleotide analogue for gene therapy.		

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1 SULPHUR CONTAINING DINUCLEOTIDE PHOSPHORAMIDITES

2
3 The present invention relates to dinucleotide
4 phosphoramidites having a non-bridging sulphur group
5 attached to the phosphorus moiety, the synthesis of
6 these compounds and their use in the synthesis of
7 phosphorothioate oligonucleotides.
8

9 The standard methodology for oligonucleotide synthesis
10 relies upon solid phase chemistry. In a typical
11 synthetic protocol phosphoramidites are added in a
12 stepwise manner to an initial immobilised nucleoside,
13 with protecting and deprotecting steps as necessary in
14 each cycle. The process is now automated and is
15 normally able to produce 10^{-6} mol quantities of the
16 desired end product. A suitable methodology is
17 described by Beaucage in Methods in Molecular Biology,
18 Vol 20, Protocols for Oligonucleotides and Analogues,
19 ed Agrawal, Humana Press, Totawa, 1993, pages 33-61.
20

21 More recently, the synthesis of S-alkyl esters of 2'-
22 deoxyribonucleoside 3'-phosphorothioates has been
23 reported (see Liu et al, J. Chem. Soc. Perkin Trans 1 :
24 1685-1694 (1995)) and the use of such compounds in the
25 synthesis of oligonucleotide phosphorothioates was

1 suggested.

2
3 Phosphorothioate oligonucleotides are regarded as the
4 first generation of antisense oligonucleotide analogues
5 which have been successfully tested *in vitro* and *in*
6 *vivo* as inhibitors of gene expression (see,
7 "Oligonucleotides: Antisense Inhibitors of Gene
8 Expression", Ed. Cohen, Macmillan, London, 1989 and
9 "Prospects for Antisense Nucleic Acid Therapy of Cancer
10 and AIDS", Ed. Wickstrom, Wiley-Liss, New York, 1992).
11 At present, a few uniformly modified phosphorothioate
12 oligonucleotides are in human clinical trials and have
13 the potential to be used as approved drugs. (see,
14 Ravikumar *et al*, Bioorganic & Medicinal Chemistry
15 Lett.: 2017-2022 [1994]). Large quantities, multiple
16 gram to multiple kilogram, of high purity
17 phosphorothioate oligonucleotides are required at low
18 and acceptable cost suitable for therapeutic
19 applications.

20
21 Phosphorothioate oligonucleotides are isoelectronic
22 analogues of natural oligonucleotides in which one of
23 the non-bridging internucleotide oxygen atoms is
24 replaced by a sulphur atom. The solid phase synthesis
25 of phosphorothioate oligonucleotides has been achieved
26 using H-phosphonate chemistry (see, Froehler *et al*,
27 Tetrahedron Lett. 5575-5578 [1986]) where only one
28 sulphur transfer step is required after assembling the
29 desired sequence to convert all the internucleotide
30 linkages to phosphorothioates, or the phosphoramidite
31 approach (see, Stec *et al*, J. Am. Chem. Soc., 6077-6079
32 [1984] and Rao *et al*, Tetrahedron Lett., 6741-6744
33 [1994]) where monomeric phosphoramidites are added in
34 each synthetic cycle and a stepwise sulphurisation
35 instead of iodine oxidation step in an otherwise
36 standard synthetic cycle is used to assemble the

1 desired phosphorothioate oligonucleotides. The solid
2 phase monomeric phosphoramidite chemistry is routinely
3 used to synthesize phosphorothioate oligonucleotides
4 (on micromole to millimole scale) as considerable
5 efforts have been expended in enhancing the efficiency
6 of the synthesis such as (i) the use of improved
7 synthetic cycle protocols and solid supports (see,
8 Ravikumar *et al*, *Bioorganic & Medical Chemistry Lett.*,
9 2017 [1994]) (ii) sulphur transfer reagents (see Rao *et al*,
10 *Tetrahedron Lett.*, 6741 (1994) and references cited
11 therein), (iii) capping and deblocking reagents (see,
12 Agrawal *et al*, *Tetrahedron Lett.*, 8565 [1994]).
13 However, problems still remain both in terms of
14 consistent yields and quality of the final
15 oligonucleotide phosphorothioate. In particular the n-
16 1 and n+1 impurities are very similar to the full
17 length product "n" and vary from batch to batch,
18 especially when reduced excesses of monomeric
19 nucleoside phosphoramidite synthons are used in each
20 synthetic cycle. In order to meet the quality
21 specifications of the full length phosphorothioate
22 oligonucleotide needed for therapeutic applications,
23 which are very high, it is necessary to repeatedly
24 purify the product, free from n-1 and n+1 impurities.
25 Consequently the process will result in lowering the
26 yield of the full length product and hence the overall
27 process might not be cost effective.

28
29 Whilst the potential utility of phosphorothioates has
30 been recognised there still remains a need for an
31 effective and efficient manufacture of these complex
32 molecules. In particular it has not previously been
33 recognised that dimeric or larger phosphoramidite
34 blockmers could be advantageously applied in their
35 synthesis via solid phase chemistry.

36

1 In order to alleviate some of these problems, recent
2 efforts have been focused on investigating the
3 feasibility of the large scale synthesis of
4 phosphorothioate oligonucleotides by the
5 phosphotriester approach in solution (see Reese *et al*,
6 J. Chem. Soc. Perkin Trans., 1685 [1995] and Imbach *et*
7 *al*, Antisense Res. Dev. 39 [1995]. While this approach
8 offers definite advantages over the solid phase
9 monomeric phosphoramidite chemistry, in that:

10

11 (i) it is more suitable for scale-up for synthesis in
12 much larger quantities, (e.g. millimoles to mole +
13 scale)

14

15 (ii) it allows addition of two or more nucleotide
16 residues at a time (i.e., block synthesis)

17

18 (iii) it offers the choice of purifying fully
19 protected blockmers at different stages prior to
20 assembling the desired sequence and

21

22 (iv) it allows much easier purification of the final
23 product,

24

25 it requires further development.

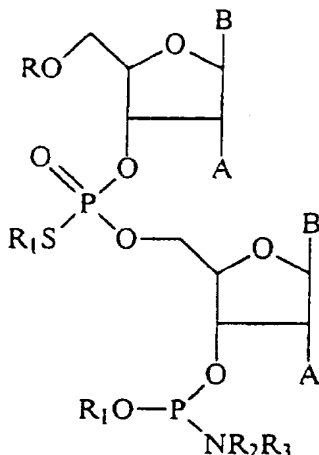
26

27 However, the solid phase phosphoramidite approach
28 (useful for micromole to millimole scale synthesis) can
29 be improved by the addition of a dimeric
30 phosphoramidite synthon instead of a monomeric
31 phosphoramidite synthon during the synthetic cycle and
32 this forms the basis of the present invention. The
33 dimeric phosphoramidite approach would achieve an
34 increased yield (as the number of steps required to
35 produce a particular oligonucleotide will be reduced)
36 and enhanced separation of the desired oligonucleotide

from the impurities (as their use results in n-2 and n+2 impurities instead of n-1 and n+1 impurities) due to the greater difference in size.

The present invention provides an improved process for the solid phase synthesis of phosphorothioate oligonucleotides using dinucleotide phosphoramidite synthons containing the S-protected phosphorothioate ester internucleotide linkage and a 3'-phosphoramidite functional group.

The present invention provides novel compounds of formula I



wherein

B represents a heterocyclic amine base or a derivative thereof;

R represents an acid labile protecting group;

R₁ represents a protecting group, preferably selected from the group consisting of 2-cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl and 4-nitrophenyl;

1 R_2 represents a blocking or protecting group;

2

3 R_3 represents a blocking or protecting group; and

4

5 A represents a hydrogen atom, or an alkoxy, allyloxy or
6 suitably protected hydroxy group.

7

8 The dinucleotide phosphoramidite of formula I can be
9 used in conventional automated solid phase synthesis to
10 produce phosphorothioate oligonucleotides.

11

12 Thus, the present invention also provides a process for
13 producing an oligonucleotide having at least one
14 phosphorothioate linkage, said process comprising
15 providing a compound of formula I above for reaction
16 with the terminal nucleoside of the nucleotide chain
17 located at the solid phase to assemble the nucleotide
18 chain. As used herein the term "nucleotide chain"
19 includes a single nucleoside located at the solid phase
20 which will itself be the terminal group available for
21 reaction.

22

23 Group R is desirably 4,4'-dimethoxytrityl, but any
24 other suitable protecting group may also be used.

25

26 Groups R_2 and R_3 may each independently be an alkyl or
27 aryl group.

28

29 The heterocyclic base of group B may be, for example a
30 purine, such as adenine, guanine or derivatives
31 thereof, or a pyrimidine, such as cytosine, uracil,
32 thymine or derivatives thereof. As derivatives may be
33 mentioned alkylated derivatives (especially methylated
34 derivatives) and halogenated derivatives, but are not
35 specially limited thereto. Uracil and derivatives
36 thereof may be especially convenient for use.

- 1 The present invention will now be further described
- 2 with reference to the following non-limiting Examples.

1 Example 1a

2
3 Triethylammonium salt of 5'-O-(4,4'-
4 dimethoxytrityl)thymidine S-(2-cyanoethyl)
5 3'-phosphorothioate (see Reese et al, J. Chem. Soc.
6 Perkin Trans. 1: 1685 [1995])
7

8 To a stirred solution of 1,2,4-triazole (8.28g, 0.126
9 mol) in anhydrous tetrahydrofuran (250ml) was added
10 triethylamine (18.08ml, 0.13 mol) and phosphorus
11 trichloride (3.5ml, 40 mmol) at approximately -35°C
12 (methanol-CO₂ bath). The reaction was stirred for 15
13 minutes, after which 5'-O-(4,4'-dimethoxytrityl)
14 thymidine (5.546g, 10.2 mmol) in tetrahydrofuran
15 (200ml) was added. After a further 30 minutes,
16 triethylamine - water (60ml, 1:1 v/v) was added
17 dropwise with stirring and the reaction mixture was
18 allowed to warm up to ambient temperature. The solvent
19 was removed under reduced pressure. The residue was
20 dissolved in chloroform (500ml) and washed with 0.5M
21 triethylammonium bicarbonate (2 x 250ml). The organic
22 layer was dried (MgSO₄) and evaporated. The residue was
23 co-evaporated with acetonitrile (3 x 100ml), and then
24 dissolved in anhydrous dichloromethane (180ml). N-(2-
25 Cyanoethylthio)phthalimide (3.09g, 13.3 mmol) was
26 added, followed by N-methylmorpholine (6.67ml, 60 mmol)
27 and chlorotrimethylsilane (5.07ml, 40 mmol). The
28 mixture was allowed to stir at ambient temperature.
29 After 3 hours, the reaction mixture was poured into
30 0.5M triethylammonium bicarbonate (200ml). The organic
31 layer was separated and the aqueous layer was extracted
32 with dichloromethane (200ml). The combined organic
33 layers (dried over MgSO₄) were evaporated. The residue
34 was purified by short-column chromatography and the
35 product-containing fractions, which were eluted with
36 CHCl₃-MeOH (90:10 to 85:15 v/v), were evaporated under

1 reduced pressure. The residue was dissolved in
2 chloroform (40ml) and the title compound was obtained
3 by precipitation from petroleum ether (b.p. 30-40°C,
4 400ml) as a colourless solid (8.10g).
5
6 δ_H [CD_3] $_2$ SO]: 1.18 (1.5 H, t, J = 7.3 Hz), 1.36 (3 H,
7 s), 2.40 (2 H, m), 2.69 (2 H, m), 2.83 (2 H, m), 3.03
8 (1 H, q, J = 7.2 Hz), 3.17 (1 H, m), 3.32 (1 H, m),
9 3.74 (6 H, s), 4.19 (1 H, m), 4.91 (1 H, m), 6.23 (1 H,
10 t, J = 7.2 Hz), 6.89 - 7.41 (13 H, m), 7.52
11 (1 H, s) 11.40 (1 H, s).
12
13 δ_P [CD_3] $_2$ SO]: 13.9 ppm
14 HPLC data: R_1 = 9.65 minute (Programme 1)
15 Column : ODS 5 μ (5 x 250 mm)
16 Eluting Conditions : Curve Select : linear gradient,
17 time of programme = 10 minutes; flow : 1.5 ml/minute;
18 Initial conditions: 0.1M triethylammonium acetate
19 (TEAA) buffer : acetonitrile (7:3, v/v)
20 Final conditions: 0.1M TEAA buffer : acetonitrile
21 (2:8, v/v)

1 Example 1b

2

3 Triethylammonium salt of N-benzoyl-5'-O-
4 (dimethoxytrityl)deoxycytidine S-(2-cyanoethyl) 3'-
5 phosphorothioate

6

7 This compound was prepared on the same scale and in
8 precisely the same way as the thymidine derivative
9 described above. N-benzoyl-5'-O-(dimethoxytrityl)
10 deoxycytidine (6.336g, 10 mmol) was converted into the
11 title compound (8.84g) as a colourless solid.

12

13 δ_H [CD_3]₂SO]: 1.19 (6 H, t, J = 7.3 Hz), 1.36 (3 H, s),
14 2.32 (1 H, m), 2.68 (1 H, m), 2.85 (2 H, m), 3.06 (4 H,
15 q, J = 7.3 Hz), 3.41 (2 H, m), 3.75 (6 H, m), 4.29 (1
16 H, m), 4.85 (1 H, m), 6.18 (1 H, t, J = 6.3 Hz), 6.90 -
17 8.00 (19 H, m), 8.18 (1 H, d, J = 7.5 Hz) 11.31 (1 H,
18 s).

19

20 δ_P [CD_3]₂SO]: 13.2 ppm

21 HPLC data: R_1 = 11.25 minutes (Programme 1)

1 Example 1c

2

3 5'-O-(Dimethoxytrityl) thymidin-3'-yl-N-
4 benzoyldeoxycytidin-5'-yl S-(2-cyanoethyl)
5 phosphorothioate

6

7 A solution of triethylammonium salt of 5'-O-
8 (dimethoxytrityl)thymidine-S-(2-cyanoethyl)-3'-
9 phosphorothioate (2.012g, 2.5 mmol) (from Example 1a),
10 N-benzoyldeoxycytidine (1.035g, 3.125 mmol) and 3-
11 nitro-1,2,4-triazole (0.998g, 8.75 mmol) in pyridine
12 (25 ml) was concentrated to dryness under reduced
13 pressure. This process was repeated twice more and the
14 residue was dissolved in dry pyridine (20ml).
15 Mesitylene-2-sulfonyl chloride (1.64g, 7.5 mmol) was
16 added and the solution was allowed to stir for 30
17 minutes. The reaction was quenched with saturated
18 aqueous sodium bicarbonate (2.5ml), and the products
19 were partitioned between chloroform (50ml) and
20 saturated aqueous sodium bicarbonate (150ml). The
21 organic layer was separated and the aqueous layer was
22 extracted with chloroform (4 x 30ml). The combined
23 organic layers were dried (MgSO₄) and evaporated under
24 reduced pressure. The residue was co-evaporated with
25 toluene (2 x 20ml) and then purified by short-column
26 chromatography. The appropriate fractions, eluted with
27 CHCl₃-MeOH (98:2 to 96.5-3.5 v/v) were combined and
28 evaporated under reduced pressure. A solution of the
29 residue in chloroform (10ml) was added dropwise to
30 petroleum ether (b.p. 30-40°C, 200ml) to give the title
31 compound as a precipitate (1.57g, 61.8%).

32

33 δ_H [(CD₃)₂SO]: 1.45 (3 H, s), 2.15 (1 H, m), 2.35 (1 H,
34 m), 2.57 (2 H, m), 2.90 (2 H, m) 3.10 (2 H, m), 3.31 (2
35 H, m), 3.73 (6 H, s), 4.07 (1 H, m), 4.23 (2 H, m),
36 4.32

1 (2 H, m), 5.23 (1 H, m), 5.56 (1 H, , d, J = 4.3 Hz),
2 6.16 (1 H, m), 6.25 (1 H, m),
3 6.87 - 8.00 (20 H, m), 8.15 (1 H, m), 11.27 (1 H, s),
4 11.41 (1 H, s).
5
6 On treatment with D₂O signals at 11.27, 11.41, 5.56 ppm
7 diminished in intensity.
8 δ_H [CD₃)₂SO]; 27.7, 28.0 ppm
9 HPLC data: R₁ = 12.12 minutes, 12.27 minutes (programme
10 1)

Example 1d

N-benzoyl-5'-O-(dimethoxytrityl)deoxycytidin-3'-yl
thymidin-5'-yl S-(2-cyanoethyl) phosphorothioate

A solution of the triethylammonium salt of N-benzoyl-5'-O-(dimethoxytrityl)deoxycytidine S-(2-cyanoethyl) 3'-phosphorothioate (4.42g, 5 mmol) (from Example 1b), thymidine (1.519g, 6.25 mmol) and 3-nitro-1,2,4-triazole (2.00g, 17.5 mmol) in dry pyridine (20ml) was concentrated to dryness under reduced pressure. This process was repeated twice more and the residue was dissolved in dry pyridine (50ml). Mesitylene-2-sulfonyl chloride (3.28g, 15.0 meal) was added and the solution was allowed to stir for 30 minutes. The reaction was quenched with saturated aqueous sodium bicarbonate (me) and the products were partitioned between chloroform (100ml) and 0.5M triethylammonium bicarbonate (200ml). The organic layer was separated and the aqueous layer was extracted with chloroform (3 x 50ml). The combined organic layers were dried (MgSO₄) and evaporated under reduced pressure. The residue was co-evaporated with toluene (3 x 20ml) and then purified by short-column chromatography. The appropriate fractions, eluted with CHCl₃-MeOH (98:2 to 97:3 v/v) were combined and evaporated under pressure. A solution of the residue in chloroform (15ml) was added dropwise to petroleum ether (b.p. 30-40°C, 300ml) to give the title compound as a precipitate (3.06g, 60%).

δ_H [CD₃)₂SO]: 1.79 (3 H, s), 2.15 (2 H, m), 2.48 (1 H, m), 2.79 (2 H, m), 2.90 (2 H, m) 3.00 (2 H, m), 3.38 (2 H, m), 3.74 (6 H, s), 3.99 (1 H, m), 4.34 (4 H, m), 5.15 (1 H, m), 5.52 (1 H, d, J = 4.5 Hz), 6.19 (2 H, m), 6.89 - 8.03 (20 H, m), 8.18 (1 H, d, J = 7.4 Hz), 11.32

1 (1 H, z), 11.35 (1 H, s).

2

3 On treatment with D₂O signals at 5.52, 11.32 and 11.35
4 ppm diminished in intensity.

5 δ_p [CD₃]₂SO}; 27.7, 27.9 ppm

6 HPLC data: R₁ = 13.00 minutes, 13.13 minutes (Programme
7 1)

1 Example 2a

2

3 5'-O-(Dimethoxytrityl)-thymidin-3'-yl-3'-[(2-S-
4 cyanoethyl)phosphoryl]-5'-N-benzoyl-2'-deoxycytidine-
5 3'-[(2-cyanoethyl)-N,N-diisopropyl] phosphoramidite

6

7 **Abbreviation:** T-P(s)-dC-CEPA

8

9 5'-O-(Dimethoxytrityl)thymidin-3'-yl N-
10 benzoyldeoxycytidin-5'-yl S-(2-cyanoethyl)
11 phosphorothioate (8.20g, 8.151 mmol, 1 mol eq) (from
12 Example 1c) was dissolved in dry dichloromethane (AR
13 grade) (120ml) under an argon blanket, and allowed to
14 stir for 5 minutes. To this solution was added
15 diisopropyl-ammonium tetrazolide (1.394g, 1 mol eq)
16 followed by bis-(N,N-diisopropylamino)-(2-O-cyanoethyl)
17 phosphoramidite (4.914g, 2 mol eq) and the reaction
18 mixture allowed to stir under an argon blanket for 1.5
19 hours. The reaction was then washed with water (75ml),
20 saturated NaCl solution (75ml) and saturated NaHCO₃
21 (75ml). The organic layers were separated and the
22 aqueous layers were back extracted with dichloromethane
23 (25ml) and the extract was added to the organic layers,
24 which were then dried over anhydrous sodium sulphate
25 (50g), filtered and then evaporated to a foam. The
26 foam was then dissolved in dichloromethane (20ml) and
27 purified on a silica chromatography column with a
28 silica/product ration of 10:1. The column was first
29 packed with 1% pyridine in dichloromethane, then once
30 the product had been loaded onto the column it was
31 eluted with dichloromethane (100ml), MeCN (2000ml), and
32 10% MeOH in dichloromethane (250ml) to strip the
33 column. The appropriate fractions were combined and
34 evaporated under reduced pressure to a foam. The
35 product was then dissolved in dichloromethane (50ml)
36 and added dropwise to pentane (500ml) to give a

1 precipitate. This was then dissolved in
2 dichloromethane and filtered through a 1 micron filter
3 system, then evaporated to a foam and placed onto a
4 freeze drier for a minimum of 8 hours. Yield = 7.5g,
5 79.3%. δ_p [CDCl₃]: 26.85, 148.91, 149.52 ppm.
6
7 Analytical data from the compound formed is presented
8 in Fig 1.

1 Example 2b

2

3 5'-O-(Dimethoxytrityl)-N-benzoyl-2'-deoxycytidine-3'-
4 yl-3'-[2-S-cyanoethyl) phosphoryl]-5'-thymidine-3'-[2-
5 cyanoethyl)-N,N-diisopropyl] phosphoramidite

6

7 Abbreviation: dC-P(S)-T-CEPA

8

9 5'-O-(Dimethoxytrityl)-N-benzoyl-deoxycytidin-3'-yl
10 thymidin-5'-yl S-(2-cyanoethyl) phosphorothioate
11 (8.00g, 7.952 mmol, 1 mol eq) (from Example 1d) was
12 dissolved in dry dichloromethane (AR grade) (120ml)
13 under an argon blanket and allowed to stir for 5
14 minutes. To this solution was added
15 diisopropylammonium tetrazolide (1.36g, 1 mol eq)
16 followed by bis(N,N-diisopropyl-amino)-(2-O-cyanoethyl)
17 phosphoramidite (4.794g, 2 mol eq), and the reaction
18 mixture was allowed to stir under an argon blanket for
19 1.5 hours. The reaction was then washed with water
20 (75ml), saturated NaCl solution (75ml). The organic
21 layers were separated and the aqueous layers were back
22 extracted with dichloromethane (25ml) and the extract
23 was added to the organic layers, which were then dried
24 over anhydrous sodium sulphate (50g), filtered, and
25 then evaporated to a foam. The foam was then dissolved
26 in dichloromethane (20ml) and purified on a silica
27 chromatography column with a silica/product ratio of
28 10:1. The column was first packed with 1% pyridine in
29 dichloromethane, then once the product had been loaded
30 onto the column it was eluted with dichloromethane
31 (100ml), MeCN (1000ml), and 10% MeOH in dichloromethane
32 (250ml) to strip the column. The appropriate fractions
33 were combined and evaporated under reduced pressure to
34 a foam. The product was then dissolved in
35 dichloromethane (50ml) and added dropwise to pentane
36 (500ml) to give a precipitate. This was then dissolved

1 in dichloromethane and filtered through a 1 micron
2 filter system, then evaporated to a foam and placed
3 onto a freeze drier for a minimum of 8 hours. Yield =
4 7.00g 73.0%. δ_p [CDCl_3]: 26.83, 149.09, 149.23 ppm

1 Example 3

2

3 Automated solid-phase synthesis of phosphorothioate
4 oligonucleotides

5

6 Synthesis of phosphorothioate oligonucleotides were
7 carried out using a Cruachem PS250 DNA/RNA synthesizer.
8 Cruachem standard DNA phosphoramidites and reagents
9 were used unless otherwise stated. One μm
10 phosphorothioate synthetic cycle protocol in
11 conjunction with a solution of 0.05M Beaucage reagent
12 [3H-1,2-benzodithiol-3-one-1,1-dioxide] with 60 seconds
13 reaction time for thiolation was used.

14

15 To evaluate the potential use of the present invention
16 for the synthesis of phosphorothioate oligonucleotides,
17 stringent coupling reaction conditions on the use of
18 phosphoramidite synthons (3-4 excess molar equivalents)
19 in conjunction with controlled pore glass containing a
20 higher nucleoside loading (100 μm /gram) were used. The
21 compounds formed in Examples (2a) and (2b) were used as
22 the corresponding solutions in anhydrous CH_3CN (0.1M).

23

24 To demonstrate the improvements of the present
25 invention, a few phosphorothioate oligonucleotides were
26 synthesized using the monomeric phosphoramidite
27 synthons and the aforesaid conditions. Identical
28 phosphorothioate oligonucleotide sequences were
29 synthesized using the dimeric phosphoramidite synthons
30 and after appropriate deprotection steps, the resulting
31 oligonucleotides were compared.

1 Oligonucleotide sequences:

2

3 Seq 1D Nos 1 & 4 : (TC)₁₀T - 21 mer

4 Seq 1D Nos 2 & 5 : (CT)₁₀T - 21 mer

5 Seq 1D Nos 3 & 6 : TCC TTC TCT CCT CTC TTC CTA -
6 21 mer

7

8 Synthesis of Seq 1D Nos 1-3

9

10 The Sequences were produced using monomeric
11 phosphoramidite synthons. The synthesis protocol
12 therefore required 20 synthesis cycles and 20
13 sulphurisation steps.

14

15 *ACE = > 98%
16 (based on DMT cation assay)

17

18 Synthesis of Seq 1D Nos 4-6

19 The Sequences were produced using the dimeric
20 phosphoramidite synthons (T-P(s)-dc-CEPA and
21 dc-p(s)-T-CEPA). The synthesis protocol therefore
22 required 10 synthesis cycles and 10 sulphurisation
23 steps.

24

25 *ACE = > 98%
26 (based on DMT cation assay)

27

28 * *Average coupling efficiency*

29

30 Deprotection of Oligonucleotide Sequences:

31 (a) Seq 1D Nos 1 to 3 synthesized using monomeric
32 phosphoramidite synthons were released from the
33 solid support and deprotected by treating with
34 concentrated aqueous ammonia (1.0mL) at 55°C for
35 12 hours. The ammoniacal solution was evaporated
36 to a pellet under reduced pressure and the
37 unpurified (crude) oligonucleotides were analysed.

1 oligonucleotide with anhydrous pyridine (1.0 mL)
2 using vacuum centrifugation. Once dried, the
3 material was treated with a solution of DBU (1,8-
4 Diazabicyclo[5, 4,0]-undec-7-ene) in anhydrous
5 pyridine (5:95, v/v 1.0ml) for 2 hours at 30°C.
6 The solvents were then removed and the residue was
7 then treated with concentrated aqueous ammonia
8 (1.0ml) at 55°C for 12 hours. The ammoniacal
9 solution was evaporated to a pellet under reduced
10 pressure and the unpurified (crude)
11 oligonucleotides were analysed.

12
13 HPLC (Ion Exchange) analysis:

14
15 Ion-exchange HPLC analysis of phosphorothioate
16 oligodeoxy-nucleotides was carried out using a Gilson
17 712 Gradient system with dual pumps and fitted with a
18 Gilson 117 UV Detector (280nm). A 5 micron Nucleopac
19 PA100 column (5 x 250 mm) was used with eluents [A] :
20 20 mM Tris-HCl buffer, pH = 8.0 and [B] : 400 mM sodium
21 perchlorate in buffer [A].

22
23 The results are shown in Figs 2 to 4.

24
25 Fig 2 shows a comparison of anion-exchange (NucleoPac
26 PA-100) chromatograms of unpurified 5'-O-DMT-on
27 phosphorothioate oligomers (TC)₁₀T 21-mer (Seq 1D Nos 1
28 and 4). Fig 2A gives the results for the 21-mer
29 synthesised with monomeric phosphoramidites (Seq 1D No
30 1) which has a product purity of 68.5%. Fig 2B gives
31 the results for the 21-mer synthesised with dimeric
32 phosphoramidites (Seq 1D No 4) which has an increased
33 product purity of 78.0%.

34
35 Fig 3 shows a comparison of anion-exchange (NucleoPac
36 PA-100) chromatograms of unpurified 5'-O-DMT-on

1 product purity of 78.0%.

2

3 Fig 3 shows a comparison of anion-exchange (NucleoPac
4 PA-100) chromatograms of unpurified 5'-O-DMT-on
5 phosphorothioate oligomers (CT)₁₀A 21-mer (Seq 1D Nos 2
6 and 5). Fig 3A gives the results for the 21-mer
7 synthesised with monomeric phosphoramidites Seq 1D No
8 2) which have a product purity of 74.0%. Fig 3B gives
9 the results for the 21-mer synthesised with dimeric
10 phosphoramidites (Seq 1D No 5) which has an increased
11 product purity of 83.0%.

12

13 Fig 4 shows a comparison of anion-exchange (NucleoPac
14 PA-100) chromatograms of unpurified 5'-O-DMT-on
15 phosphorothioate oligomers (TCC TTC TCT CCT CTC TTC
16 CTA) 21-mer (Seq 1D Nos 3 and 6). Fig 4A gives the
17 results for the 21-mer synthesised with monomeric
18 phosphoramidites (Seq 1D No 3) which have a product
19 purity of 73.8%. Fig 4B gives the results for the 21-
20 mer synthesised with dimeric phosphoramidites (Seq 1D
21 No 6) which has an increased product purity of 85.5%.

22

23 Fig 5 is a comparison of ³¹P NMR spectra of unpurified
24 5'-O-DMT-on phosphorothioate oligomers for Seq 1D Nos 3
25 and 6.

26

27 A: synthesised using monomeric phosphoramidites (Seq
28 1D No 3)

29 B: synthesised using S-dimeric phosphoramidites (Seq
30 1D No 6).

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT:

(A) NAME: CRUACHEM LTD

(B) STREET: WEST OF SCOTLAND SCIENCE PARK, TODD CAMPUS,
ACRE ROAD

(C) CITY: GLASGOW

(E) COUNTRY: UK

(F) POSTAL CODE (ZIP): G20 0UA

(ii) TITLE OF INVENTION: COMPOUNDS

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk

(B) COMPUTER: IBM PC compatible

(C) OPERATING SYSTEM: PC-DOS/MS-DOS

(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: GB 9602326.2

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

TCTCTCTCTC TCTCTCTCTC T

21

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

24

- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

CTCTCTCTCT CTCTCTCTCT A

21

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

TCCTTCTCTC CTCTCTTCCT A

21

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

- (A) NAME/KEY: modified_base
- (B) LOCATION: group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)
- (D) OTHER INFORMATION: /mod_base= OTHER
/label= PHOSPHOROTHIOAT

25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

TCTCTCTCTC TCTCTCTCTC T

21

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)

(D) OTHER INFORMATION: /mod_base= OTHER
/label= PHOSPHOROTHIOAT

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTCTCTCTCT CTCTCTCTCT A

21

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:

(A) NAME/KEY: modified_base

(B) LOCATION: group(2, 4, 6, 8, 10, 12, 14, 16, 18, 20)

(D) OTHER INFORMATION: /mod_base= OTHER
/label= PHOSPHOROTHIOAT

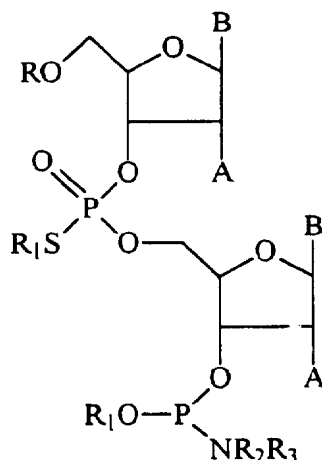
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TCCTTCTCTC CTCTCTTCT A

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1 CLAIMS

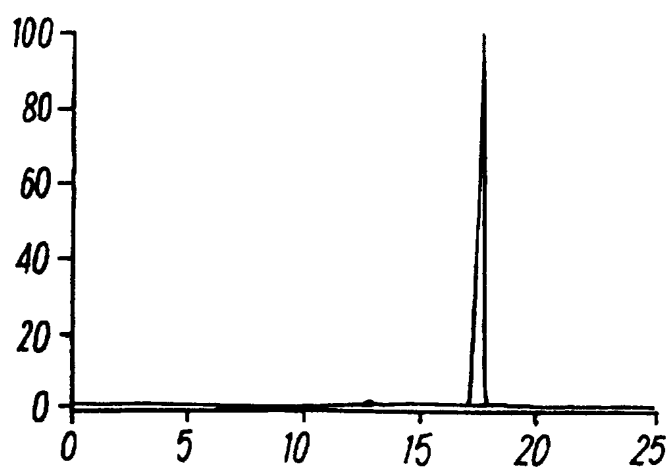
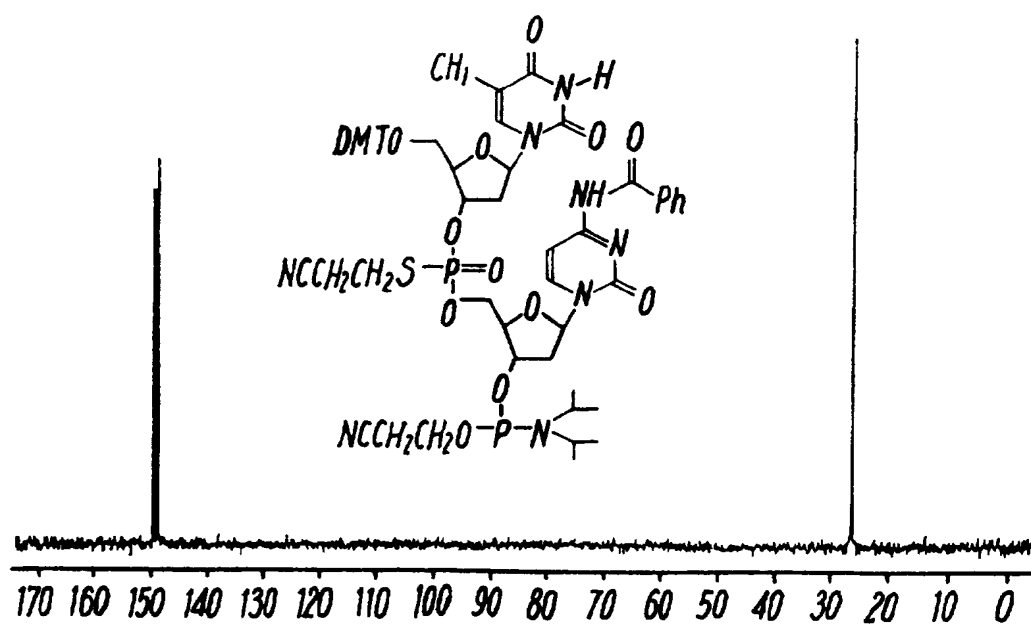
- 2
- 3 1. A process for the solid phase synthesis of
- 4 phosphorothioate oligonucleotides, said process
- 5 comprising the addition of at least one dimeric
- 6 phosphoramidite synthon during the synthetic
- 7 cycle, wherein said dimeric phosphoramidite
- 8 synthon comprises in its internucleotide linkage
- 9 an optionally protected thioester group.
- 10
- 11 2. A process as claimed in Claim 1 wherein said
- 12 dimeric phosphoramidite synthons are used as
- 13 reactants in each synthetic cycle.
- 14
- 15 3. A process as claimed in either one of Claims 1 and
- 16 2 wherein said thioester group present in said
- 17 internucleotide linkage is protected by a 2-
- 18 cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl or
- 19 4-nitrophenyl group.
- 20
- 21 4. A dimeric phosphoramidite synthon being a compound
- 22 of Formula I:
- 23



- 1 wherein
2 B represents a heterocyclic amine base or a
3 derivative thereof;
4 R represents an acid labile protecting group;
5 R₁ represents a protecting group;
6 R₂ represents a blocking or protecting group;
7 R₃ represents a hydrogen atom, or an alkoxy,
8 allyloxy or suitably protected hydroxy group.
9
- 10 5. A compound as claimed in Claim 4 wherein group B
11 is an adenine, guanine, cytosine, uracil or
12 thymine base or the alkylated or halogenated
13 derivatives of any of those bases.
14
- 15 6. A compound as claimed in Claim 5 wherein at least
16 one group B is uracil or methylated uracil.
17
- 18 7. A compound as claimed in any one of Claims 4 to 6
19 wherein group R is a 4,4'-dimethoxytrityl group.
20
- 21 8. A compound as claimed in any one of Claims 4 to 7
22 wherein each group R₁ is independently a 2-
23 cyanoethyl, 2-chlorophenyl, 2,4-dichlorophenyl or
24 4-nitrophenyl group.
25
- 26 9. A compound as claimed in any one of Claims 4 to 8
27 wherein each group R₂ and group R₃ is independently
28 an alkyl or aryl group.
29
- 30 10. Use of a compound as claimed in any one of Claims
31 4 to 9 in the synthesis of phosphorothioate
32 oligonucleotides.
33
- 34 11. Use of phosphorothioate oligonucleotides produced
35 in accordance with the process of Claims 1 to 3 as
36 anti-sense nucleotides for inhibition of gene

1 expression.

1 / 5

**FIG. 1**

2/5

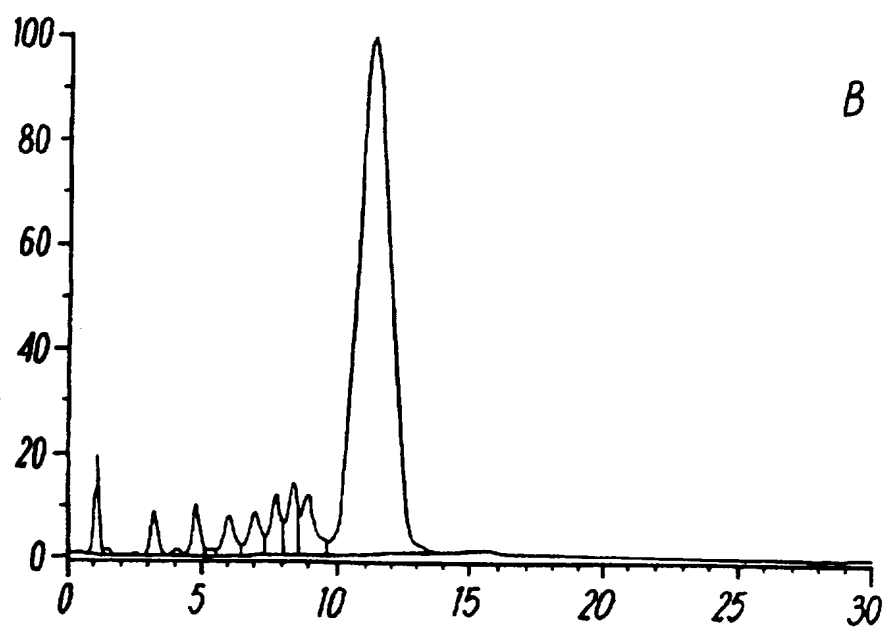
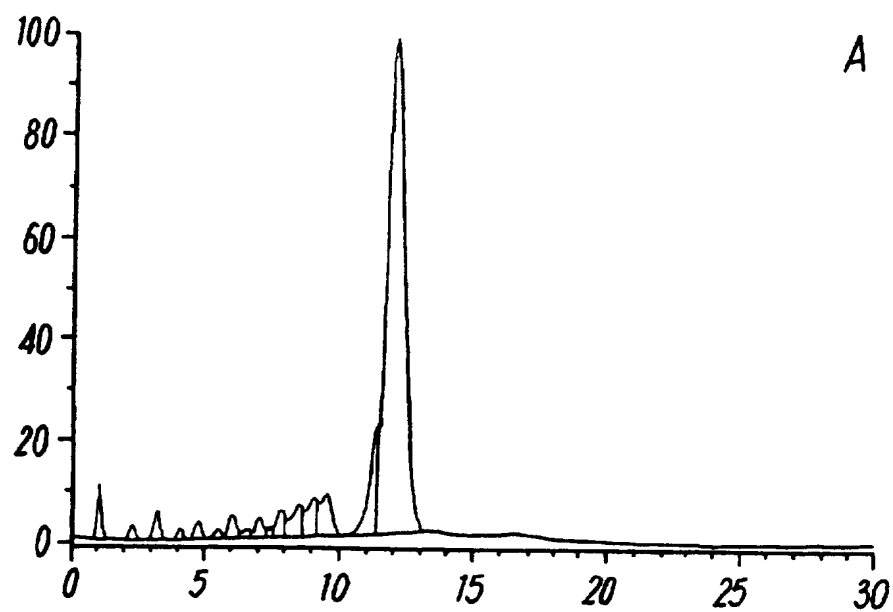
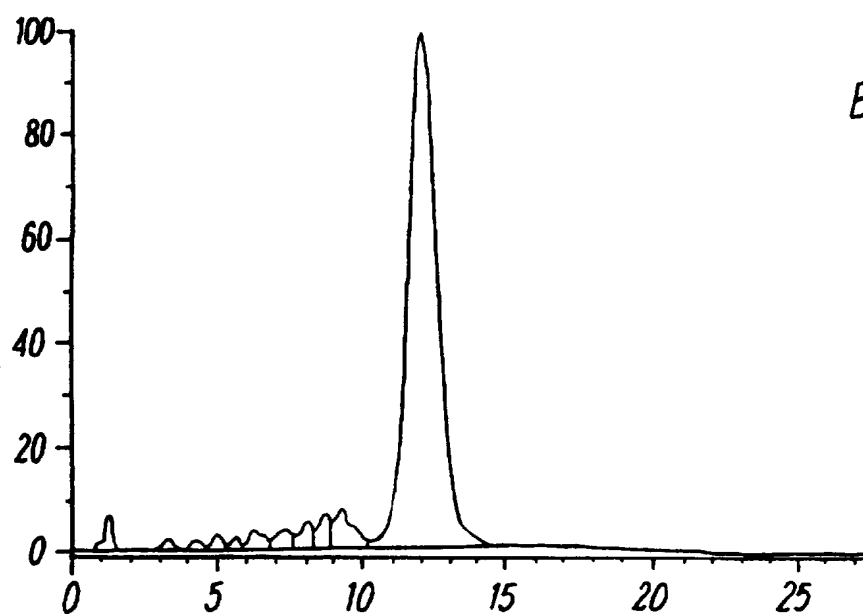
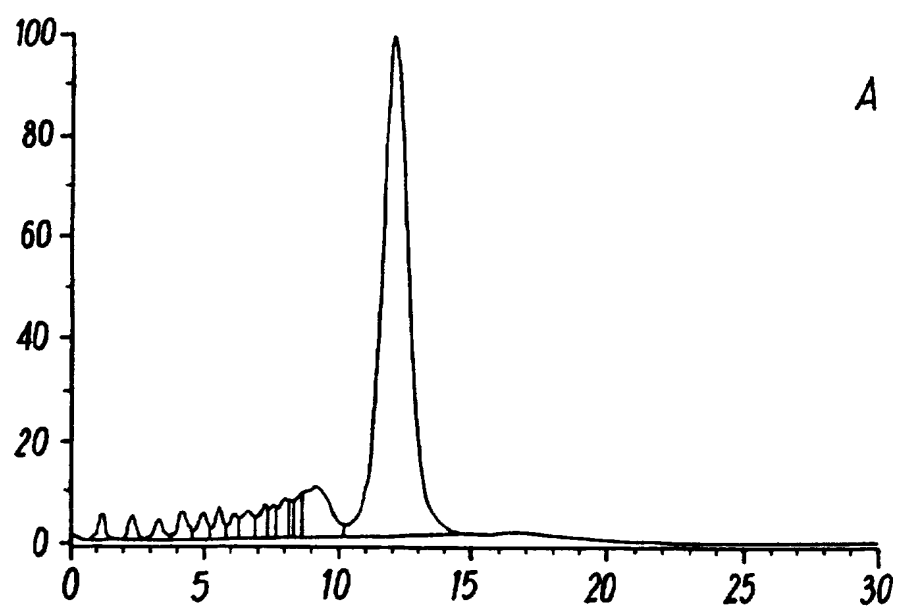


FIG. 2

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3 / 5

**FIG. 3**

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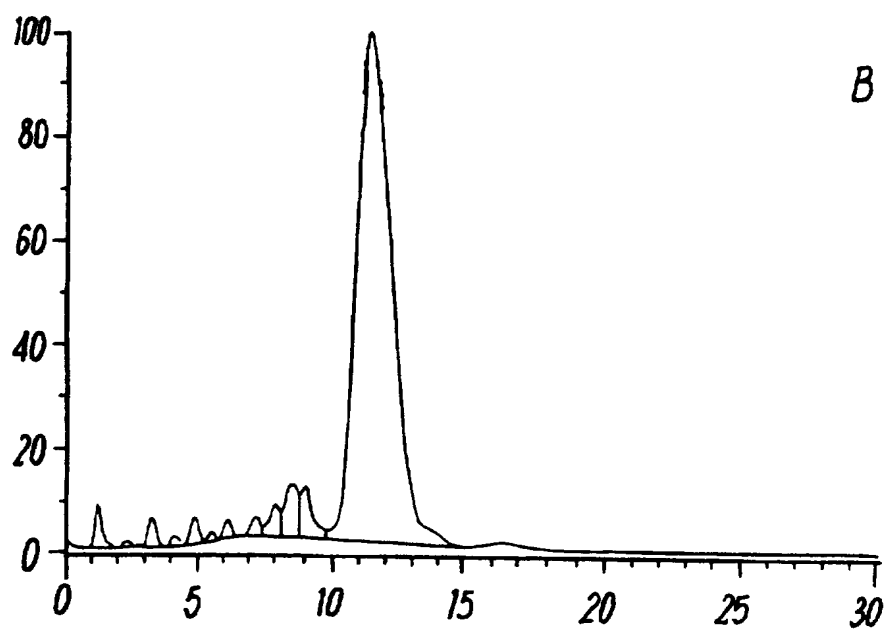
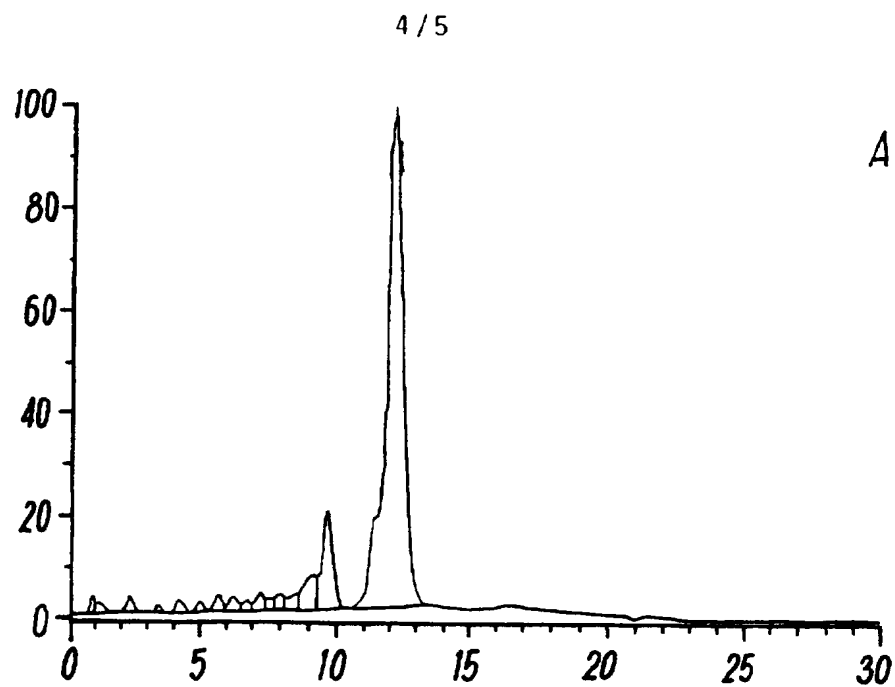
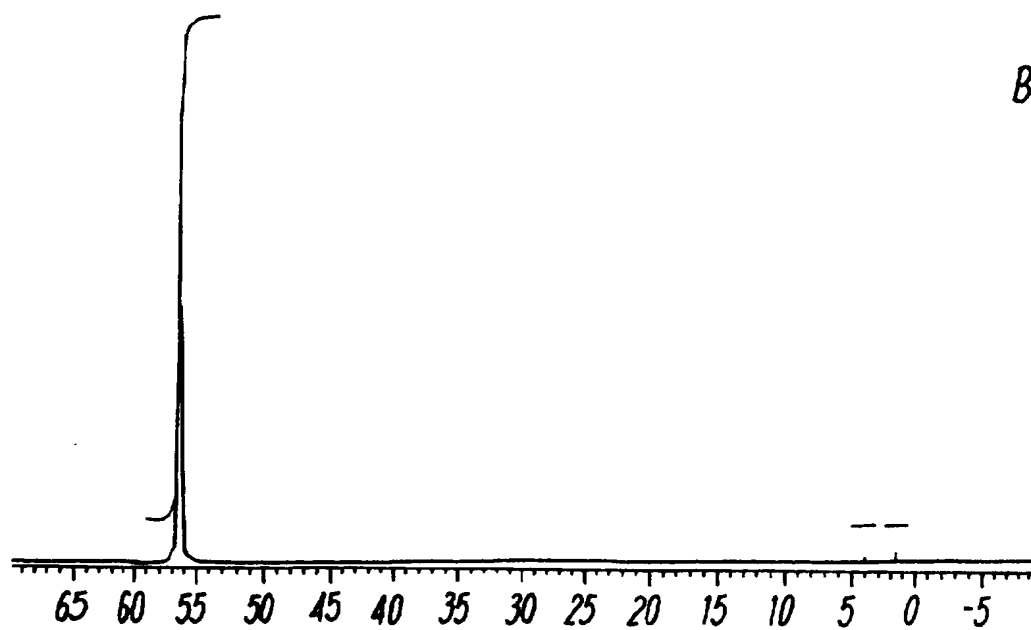
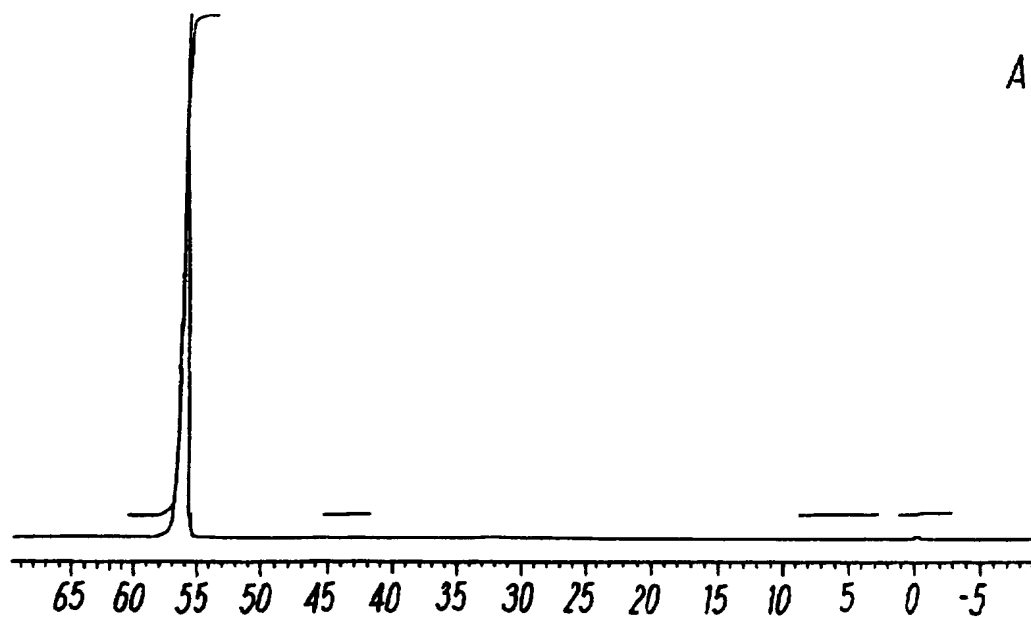


FIG. 4

5/5

**Fig. 5**

SUBSTITUTE SHEET (RULE 26)

INTERNATIONAL SEARCH REPORT

International Application No

PC1/GB 97/00327

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07H21/00 A61K31/70

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07H A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NUCLEOSIDES & NUCLEOTIDES, vol. 11, no. 9, 1 January 1992, pages 1621-1638, XP000564715 ZBIGNIEW J LESNIKOWSKI: "THE FIRST STEREOCONTROLLED SYNTHESIS OF THIOOLIGORIBONUCLEOTIDE: (RPRP)- AND (SPSP)-UPSUSU" see the whole document ---	1-11
A	WO 95 32980 A (ISIS PHARMACEUTICALS INC ;RAVIKUMAR VASULINGA (US); COLE DOUGLAS L) 7 December 1995 see the whole document ---	1,4
A	WO 95 14029 A (BECKMAN INSTRUMENTS INC) 26 May 1995 see the whole document ---	1,4
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Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

12 June 1997

Date of mailing of the international search report

24-06-1997

Name and mailing address of the ISA

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Moreno, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 97/00327

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	US 5 151 510 A (STEC WOJCIECH J ET AL) 29 September 1992 see the whole document -----	1,4

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PC 1/GB 97/00327

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